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Neuropilin-2: Novel Biomarker and Therapeutic Target for Aggressive Prostate Cancer

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14. ABSTRACT

The focus of this proposal is Neuropilin-2 (NRP2), a VEGF receptor that is not expressed in normal prostate but is expressed in prostate cancer and correlates with Gleason grade. We demonstrated that PTEN deletion induces NRP2 expression and propose that NRP2 contributes to the function of prostate cancer stem cells and tumor formation. Recently, we obtained rigorous genetic evidence implicating NRP2 in the formation of prostate tumors, and in the genesis and function of prostate cancer stem cells. We also discovered that NRP2 facilitates the expression of Bmi-1, a transcriptional repressor, and that NRP2 suppresses the IGF-1 receptor (IGF-1R) by a mechanism that involves transcriptional repression by Bmi-1. We have obtained preliminary evidence that targeting NRP2 directly on tumor cells in combination with IGF-1R inhibition is effective treating aggressive prostate carcinoma and pursuing this possibility more rigorously. Recently, we generated a model of resistance to therapy that reinforces the importance of VEGF/NRP2 signaling to the function of prostate cancer stem cells and the approach of targeting this pathway for therapy.

15. SUBJECT TERMS
Prostate cancer, VEGF, Neuropilin, IGF-1 receptor, PTEN, stem cells, therapy

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1. INTRODUCTION: This study is based on the premise that prostate carcinoma (PCa) cells express receptors for VEGF and that these receptors contribute to tumor initiation. The focus is on Neuropilin-2 (NRP2), a VEGF receptor that is not expressed in normal prostate but is expressed in PCa and correlates with Gleason grade. It is proposed that PTEN deletion induces NRP2 expression and that NRP2 contributes to PCa formation. The role of VEGF/NRP2 signaling in prostate tumorigenesis can be explained by the discovery that NRP2 facilitates the expression of Bmi-1, a transcriptional repressor that has a critical role in the function of PCa stem cells (1). We hypothesize that NRP2 suppresses the IGF-1 receptor (IGF-1R) by a mechanism that involves transcriptional repression by Bmi-1 and, as a consequence, confers resistance to IGF-1R therapy of prostate carcinoma. This hypothesis is significant because several IGF-1R inhibitors are in clinical trials (2) but the mechanisms to account for patient response to these inhibitors are largely unknown. Similarly, clinical trials of the VEGF Ab bevacizumab have been disappointing for reasons that are not entirely known (3) but it is worth noting that this drug does not inhibit the VEGF/NRP2 interaction (4). For these reasons, targeting NRP2 directly on tumor cells in combination with IGF-1R inhibition should be a novel and a potentially potent approach for treating aggressive prostate carcinoma.

2. KEYWORDS: Prostate cancer, VEGF, Neuropilin, IGF-1 receptor, PTEN, stem cells, therapy

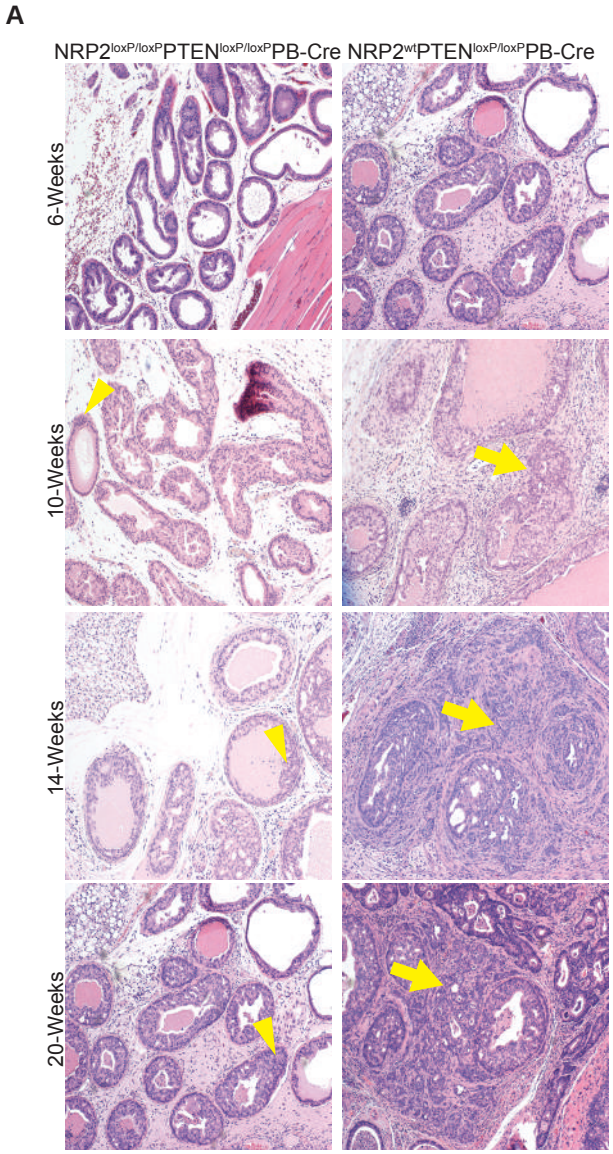

FIG. 1: Conditional deletion of NRP2 delays prostate cancer progression in PTENpc-/- mice. A. Histopathological analysis of prostate glands from NRP2loxP/loxP/PTEN loxP/loxP/PB-Cre and NRP2wt/PTEN-loxP/loxP/PB-Cre mice at various age group were stained with H&E. Arrows, adenocarcinoma; arrowheads, PIN lesions. **B.** Prostate lobes: DP: dorsal; LP: lateral; VP: ventral. Percentage of prostate glands showing hyperplasia in 6 weeks old NRP2loxP/loxP/PTEN loxP/loxP/PB-Cre and NRP2wt/PTEN-loxP/loxP/PB-Cre were counted and shown. DLP: Dorsolateral Prostate; VP: Ventral Prostate. Statistical analysis was performed using students' t-test.

3. OVERALL PROJECT SUMMARY: During the second year of this award, we have made progress on the following tasks:

Task 1. Establish that VEGF/NRP2 signaling contributes to the function of tumor-initiating cells and the formation of prostate carcinoma induced by PTEN deletion. Significant progress has been made on Task 1. The breeding events outlined in the original proposal have been completed and we have analyzed the prostate tumors that were generated. Specifically, we found that conditional deletion of NRP2 in $PTEN^{pc-/-}$ transgenic mice delays the development of prostate intraepithelial neoplasia (PIN) and prostate adenocarcinoma significantly. This conclusion was obtained by comparing age-matched $NRP2^{loxP/loxP}/PTEN^{loxP/loxP}/PB\text{-}Cre$ and $NRP2^{wt}/PTEN^{loxP/loxP}/PB\text{-}Cre$ mice. **Figure 1** reports the impact of deleting NRP2 in the $PTEN^{pc-/-}$ transgenic mice on the histology of the prostate and the development of adenocarcinoma (**Figure 1A**) and PIN (**Figure 1B**). **Table 1** quantifies these results as a function of age and number of mice examined. These results provide a rigorous validation of our hypothesis that NRP2 has an essential role in the genesis of prostate cancer. These data also complete Tasks 1a and 1b in our SOW.

Subsequently, we purified the stem-like cells [(Lin$^-$Sca-1$^+$CD49f^{high} cells), referred to as LSC cells] from the NRP2$^{loxP/loxP}$/PTEN $^{loxP/loxP}$/PB-Cre and NRP2^{wt}/PTEN$^{loxP/loxP}$/PB-Cre tumors by FACS. For this purpose, prostate tumors from 6 week old mice were excised, treated with dispase and the resulting single cell suspension was stained with lineage (Lin) markers (CD31, CD45 and Ter119), Sca-1 and CD49f. These data are reported in **Figure 2**. Our results revealed that conditional deletion of NRP2 caused a significant decrease in the frequency of LSC cells. Also, analysis of the Lin$^-$ cells indicated that NRP2 expression is essentially ablated in this population obtained from the NRP2$^{loxP/loxP}$/PTEN $^{loxP/loxP}$/PB-Cre tumors compared to the control tumors and the ability of this population to form prostatospheres is dramatically reduced. Together, these data provide convincing and rigorous evidence that NRP2 is necessary for the genesis and function of prostate cancer stem cells. These results also complete Tasks 1c and 1d in our SOW.

Phenotype	Age (weeks)	NRP2pc-/-PTENpc-/-PB-Cre	NRP2wtPTENpc-/-PB-Cre	p-value
Prostatic Intraepithelial Neoplasia (PIN)	6	1 out of 8	8 out of 8	0.001
Adenocarcinoma (WD + PD)	6	0 out of 8	1 out of 8	1
Prostatic Intraepithelial Neoplasia (PIN)	10	5 out of 9	10 out of 10	0.033
Adenocarcinoma (WD + PD)	10	0 out of 9	6 out of 10	0.011
Prostatic Intraepithelial Neoplasia (PIN)	14	8 out of 10	9 out of 9	0.47
Adenocarcinoma (WD + PD)	14	1 out of 8	8 out of 8	0.001
Prostatic Intraepithelial Neoplasia (PIN)	20	10 out o f10	7 out of 7	1
Adenocarcinoma (WD + PD)	20	2 out of 9	7 out of 7	0.003

Table 1: Histopathological analysis. The table shows the incidence (number of mice) of PIN (Prostatic Intraepithelial Neoplasia) or cancer (Well-differentiated adenocarcinoma and poorly-differentiated adenocarcinoma) in various age-group NRP2loxP/loxP/PTEN loxP/loxP/PB-Cre and NRP2wt/PTENlox-P/loxP/PB-Cre mice. NRP2loxP/loxP/PTEN loxP/loxP/PB-Cre mice show a statistically significant decrease in incidence of PIN and of cancer as compared to age-matched NRP2wt/PTENloxP/lox-P/PB-Cre mice. Statistical analysis was performed using Fisher's exact test.

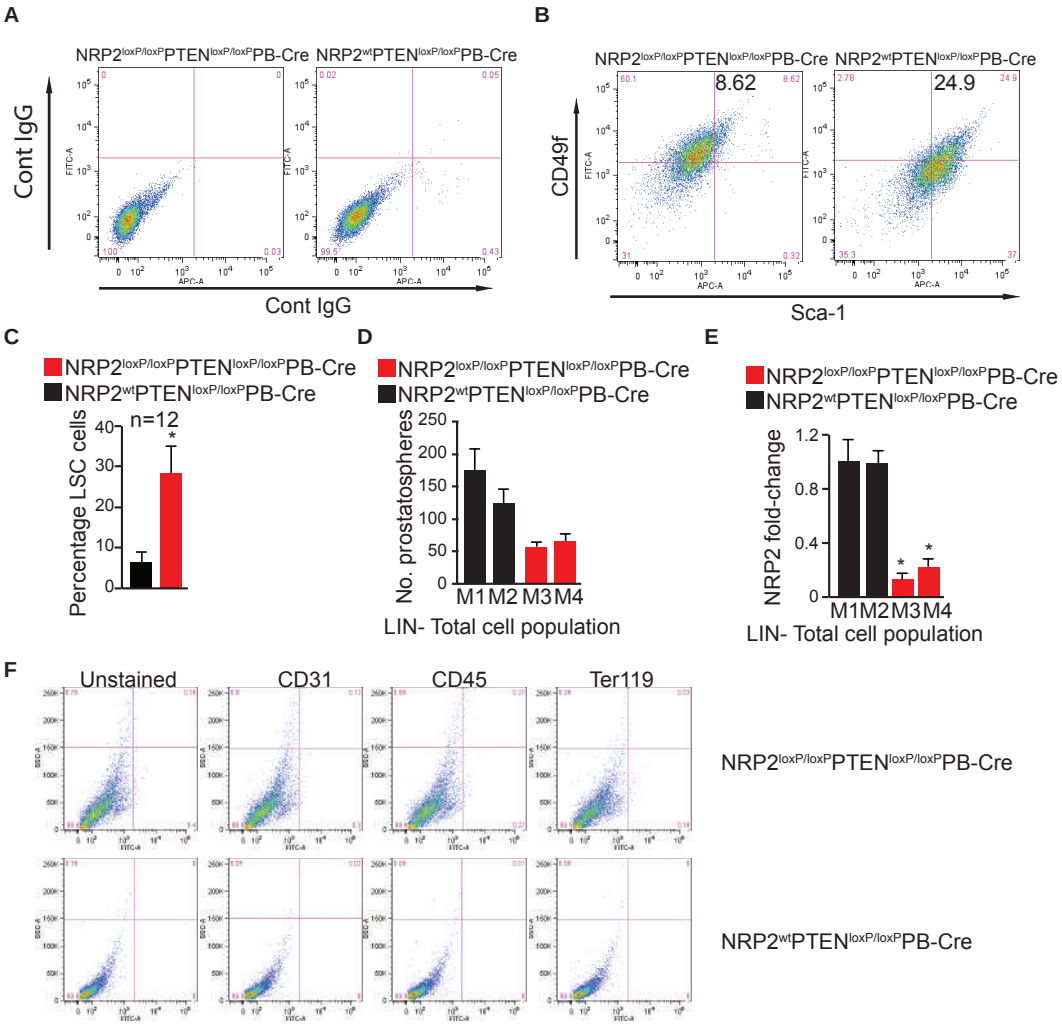

FIG. 2: Conditional deletion of NRP2 significantly reduces number of LSC stem-like cells in PTEN-pc-/- mice. A-B Prostate lobes were harvested from NRP2loxP/loxP/PTEN loxP/loxP/PB-Cre and NRP2wt/PTENloxP/loxP/PB-Cre mice (6-wks old) and digested using dispase solution at 37°C and single cell suspension were stained with lineage markers (CD31, CD45 and Ter119), Sca-1, and CD49f. Cell sorting and analysis was done using the BD FACS instrument (BD Biosciences). **C.** Average of Percentage of LSC cells from 12 mice were shown. **D.** LIN- cells from NRP2loxP/loxP/PTEN loxP/loxP/PB-Cre and NRP2wt/PTENloxP/loxP/PB-Cre mice (6-wks old) were analyzed for their ability to form prostatospheres. **E** Downregulation of NRP2 in NRP2loxP/loxP/PTEN loxP/loxP/PB-Cre mice was confirmed at RNA levels using qPCR. Panel **F** shows negative profile of lineage markers in FACS sorted LSC cells.

Task 2. Establish that NRP2 represses the IGF-1R and assess the consequences of this regulation on the function of prostate carcinoma cells. The transgenic model reported for Aim 1 was also used to establish the relationship between NRP2 and IGF-1R rigorously. For this purpose, the Lin- population isolated from the NRP2$^{loxP/loxP}$/PTEN $^{loxP/loxP}$/PB-Cre and NRP2^{wt}/PTEN$^{loxP/loxP}$/PB-Cre tumors was analyzed by immunoblotting for expression of

NRP2 and the IGF-1R. These data are reported in **Figure 3**. Specifically, we observed that conditional deletion of NRP2 induced expression of the IGF-1R. This result provides a critical validation of our hypothesis that VEGF/NRP2 signaling represses the IGF-1R and that effective therapeutic approaches need to inhibit both NRP2 and the IGF-1R. This important finding completes Task 2c in our SOW.

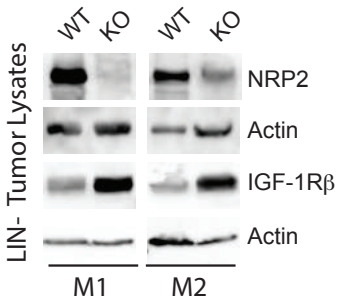

Fig. 3: Conditional deletion of NRP2 significantly increases expression of IGF-1R in PTENpc-/- mice. Prostate lobes were harvested from NRP2loxP/loxP/PTEN loxP/loxP/PB-Cre and NRP2wt/PTENloxP/loxP/PB-Cre mice (6-wks old) and digested using dispase solution at 37°C and single cell suspension were stained with lineage markers (CD31, CD45 and Ter119), Sca-1, and CD49f. Cell sorting and analysis was done using the BD FACS instrument (BD Biosciences). LIN- popultions were lysed and immunoblotted to detect the expression of IGF-1R.

Task 3. Evaluate the relationship between NRP2 and IGF-1R in PCa therapy. A central premise of Task 3 is that VEGF/NRP2 signaling is a prime target for therapy because it is essential for the function of prostate cancer stem cells (see Task 1). However, many drugs that are currently used to inhibit VEGF such as bevacizumab or its tyrosine kinase receptors do not inhibit the interaction of VEGF with NRP2. For this reason, these drugs may reduce tumor volume but they will not eradicate the cancer stem cell population resulting in resistance and relapse. To test this hypothesis, we developed a system where prostate cancer cells were exposed to these drugs until they became resistant and no longer impacted cell survival. Specifically, we used: bevacuzimab, a humanized VEGF antibody; sunitinib, an inhibitor of VEGF receptor tyrosine kinases; and pazopanib, another VEGF receptor tyrosine kinase inhibitor. We exposed PC3 and C4-2 cells, two well-established, aggressive prostate cancer cell lines, to increasing concentration of these three drugs and generated resistant cell lines (PC3-R and C4-2R), which are no longer sensitive to these inhibitors. The data reporting their resistance and sensitivity are provided in **Figure 4**. Interestingly PC3-R and C4-2R cells are also resistant to taxol. Importantly, we found that the resistant cells were able to form prostatopheres and initiate tumors in mice much better than the sensitive cells (**Figure 4**). This finding suggests that the resistant cells are enriched for prostate cancer stem cells, supporting our initial hypothesis.

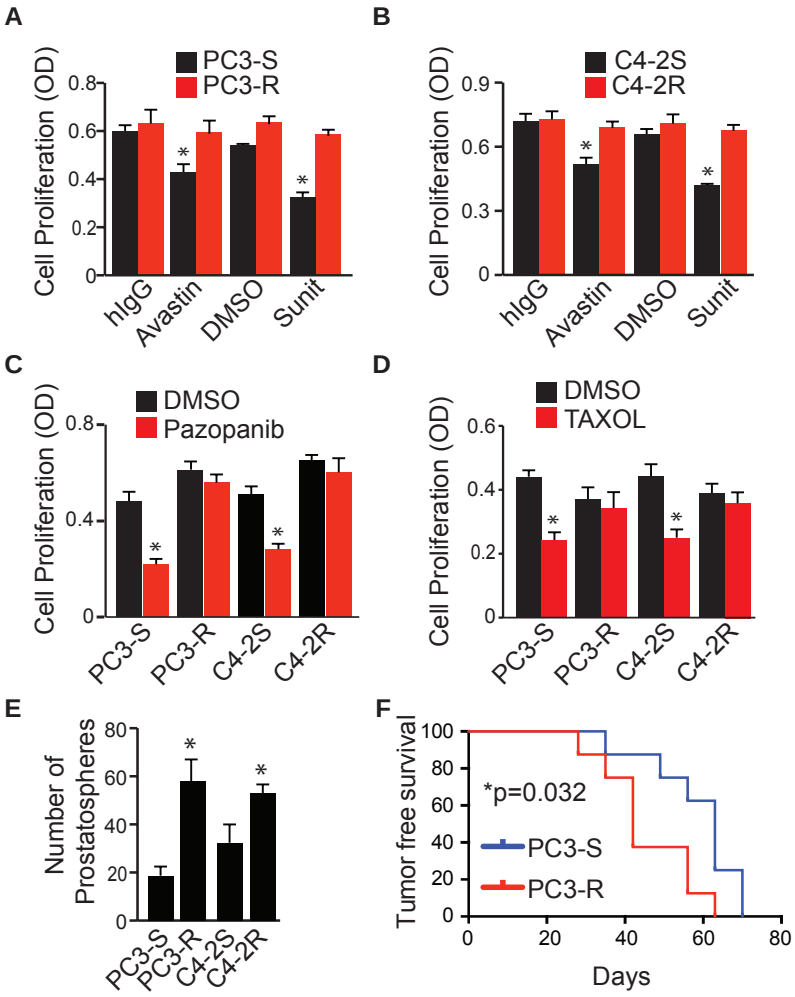

Fig. 4: Characterization of novel cell population resistant to inhibition of VEGF signaling pathways: A-B. PC3 and C4-2 cells were cultured in the presence of bevacizumab, Sunitinib or respective controls for several days with increasing concentrations. After several passage, there is development of resistant cell lines, which are resistant to 1 mg/ml of bevacizumab and 50 !M of sunitinib. **C-D.** PC3 and C4-2 resistant and sensitive cell lines were analyzed for cell proliferation in the presence or absence of 10 ! M Pazopanib or 20nM Taxol and found that resistant variants are tolerant to these drug. **E.** Resistant populations show increased ability to form prostatospheres indicating enrichment of tumor-initiating cells in these populations. **F.** Resistant and sensitive PC3 populations were implanted in immunocompromised mice and tumor onset was plotted. Results show that resistant population has high tumor onset ability compared to sensitive population.

To gain insight into the mechanism of resistance, we performed gene expression analysis on the resistant and sensitive cells.

The resistant cells were highly enriched for the expression of stem cell genes (Oct 4, BMI-1, Nanog and Sox2), VEGF and NRP2 compared to the sensitive population. These data, which are provided in **Figure 5**, strongly support our hypothesis that resistance to therapy enriches for cancer stem cells and that these cells exhibit VEGF/NRP2 signaling. In the context of Task 3, these data highlight the importance of targeting NRP2 in prostate cancer. As proof of principle, we demonstrated that c-furSEMA, an inhibitory peptide that blocks the interaction of VEGF with NRPs, inhibits prostatosphere formation in resistant cell lines and has no effect in sensitive cells (**Figure 5**).

A

	C4-2S	C4-2R	PC3-S	PC3-R
STEMNESS				
OCT 4	1 (0.03)	0.98 (0.02)	1 (0.07)	3.3 (0.06)
Bmi-1	1 (0.03)	2.9 (0.042)	1 (0.05)	7.6 (0.43)
GLI1	1 (0.07)	4.2 (0.28)	1 (0.09)	8.5 (0.57)
KLF4	1 (0.21)	1.39 (0.09)	1 (0.08)	0.91 (0.18)
NANOG	1 (0.042)	3.1 (0.04)	1 (0.03)	15 (1.99)
SOX2	1 (0.17)	4.8 (0.139)	1 (0.42)	5.6 (0.84)
ALDH1	1 (0.46)	2.2 (0.68)	1 (0.19)	1.9 (0.48)
ALDH2	1 (0.8)	1.6 (0.6)	1 (0.23)	1.15 (0.63)
ALDH3	1 (0.02)	0.9 (0.45)	1 (0.02)	1.4 (0.8)
Snail1	1 (0.1)	1.6 (0.5)	1 (0.09)	2.6 (0.12)
VEGF	1 (0.05)	8.7 (0.2)	1 (0.03)	6.3 (0.7)
GROWTH FACTOR RECEPTOR				
c-Met	1 (0.13)	1.04 (0.36)	1 (0.037)	1.17 (0.42)
EGFR	1 (0.055)	0.5 (0.02)	1 (0.01)	3.6 (1.89)
IGF1R	1 (0.035)	0.12 (0.05)	1 (0.049)	0.18 (0.07)
IR	1 (0.04)	1.23 (0.52)	1 (0.15)	2.2 (1.11)
VEGFR1	0	0	0	0
VEGFR2	1.18 (0.68)	0.68 (0.12)	1 (0.23)	1.5 (0.58)
NPR1	1 (0.02)	6.3 (0.06)	1 (0.01)	6.11 (0.44)
NPR2	1 (0.042)	17.7 (1.56)	1 (0.27)	15.08 (1.99)

B

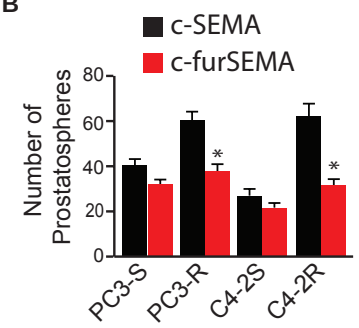

Fig. 5. Resistant populations are enriched with cancer stem cells and required neuropilins for stemness. A. Resistant and sensitive populations were compared for mRNA expression of stem cell related genes and VEGF receptors by qPCR. **B.** Resistant and sensitive populations were studied for their ability to make prostatospheres in the presence of either NRP inhibitory peptide c-furSEMA or control.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Obtained rigorous genetic evidence that NRP2 is essential for the development of PIN and adenocarcinoma in the prostate.
- Obtained rigorous genetic evidence that NRP2 is necessary for the genesis and function of prostate cancer stem cells.
- Demonstrated that the IGF1-R is induced in response to conditional deletion of NRP2 in prostate cancer, highlighting the need to target both NRP2 and the IGF1-R as an effective therapeutic strategy.
- Developed a model of resistance to therapy in prostate cancer and demonstrated that resistance increases the frequency of cancer stem cells and that these cells exhibit VEGF/NRP2 signaling.

5. CONCLUSIONS: During this year of funding, we have made significant progress in accomplishing the goals outlined in the SOW in the original proposal. The data we have obtained validate our central hypothesis that VEGF/NRP2 signaling is essential for the function of prostate cancer stem cells and tumor initiation, and that this pathway is a viable target for therapy. A major accomplishment during this period has been the generation and analysis of the transgenic models of prostate cancer that were proposed initially. This approach enabled us to obtain rigorous data to support the contribution of NRP2 to the genesis and function of LSC stem cells and tumor formation, and to provide proof of the inverse relationship between NRP2 and IGF1-R. These data support the hypothesis that two opposing growth factor pathways exist in the prostate. IGF/IGF-1R signaling maintains a differentiated state that is associated with less aggressive tumors and fewer stem cells. Conversely, VEGF/NRP2 signaling promotes de-differentiation and is characteristic of more aggressive tumors, especially because this pathway is necessary for the genesis and function of LSC stem cells.

A major goal of this proposal is to support the feasibility of targeting NRP2 and the IGF1-R for therapy, especially for aggressive prostate cancer. Our development of model of resistance to therapy strengthens our hypothesis that resistance increases the frequency of cancer stem cells and that these cells exhibit enhanced VEGF/NRP2 signaling. We are now poised to complete Tasks 2 and 3, emphasizing the therapeutic targeting of NRP2 and IGF1-R.

6. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:

Most of the current year of funding was spent generating and analyzing the transgenic mouse data, which requires considerable time. Now that these data have obtained, we are preparing a manuscript that demonstrating the importance of NRP2 to the genesis of prostate cancer stem cells and tumor formation. We are also preparing a second manuscript on the resistance model that we developed.

7. INVENTIONS, PATENTS AND LICENSES: None to report.

8. REPORTABLE OUTCOMES:

- Generation of a transgenic mouse model of prostate cancer in which NRP2 has been conditionally deleted.
- Development of a model for studying resistance to therapy in prostate cancer.

9. OTHER ACHIEVEMENTS:

- Development of prostate cancer cell lines that are either sensitive or resistant to therapy (bevacuzimab; sunitinib and pazopanib). These cell lines provide a useful system for studying resistance to therapy and the importance of VEGF/NRP2 signaling in the acquisition of stem cell properties.

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11. APPENDIX: None